1997

Advanced glycation endproducts and the development of accelerated atherosclerosis in diabetic apolipoprotein E deficient mice

Lisa Park
Yale University

ADVANCED GLYCATION ENDPRODUCTS AND THE DEVELOPMENT OF ACCELERATED ATHEROSCLEROSIS IN DIABETIC APOLIPOPROTEIN E DEFICIENT MICE

Lisa Park

Yale University

1997

Advanced Glycation Endproducts and the Development of Accelerated Atherosclerosis in Diabetic Apolipoprotein E Deficient Mice

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

Lisa Park
1997

ADVANCED GLYCATION ENDPRODUCTS AND THE DEVELOPMENT OF ACCELERATED ATHEROSCLEROSIS IN DIABETIC APOLIPOPROTEIN E DEFICIENT MICE

Lisa Park, David Stern, and Ann Marie Schmidt. Department of Physiology, Columbia College of Physicians and Surgeons, New York, NY. (Sponsored by Joseph Madri, Department of Pathology, Yale University School of Medicine)

Diabetes mellitus is associated with accelerated disease of both the micro- and macrovasculature, with the major morbidity and mortality in this population attributable to cardiovascular disease. Under hyperglycemic conditions, proteins and lipids are modified by non-enzymatic glycation, resulting in the formation and accumulation of advanced glycation endproducts, or AGEs. This heterogeneous class of compounds shares a number of characteristics including the ability to interact with specific cellular receptors, the best characterized of which is RAGE, the receptor for AGE. To investigate the possible contribution of AGEs to accelerated diabetic vascular disease, we studied apolipoprotein E deficient mice, which develop atherosclerosis on a normal chow diet. Diabetes was induced by multiple intraperitoneal injections of streptozotocin (55 mg/kg x 6 days). Sustained hyperglycemia was documented by elevated plasma glucose levels (>300 mg/dl) and a 1.8-fold increase in HbA_{1c} ($p<0.05$) in the streptozotocin-treated mice. Animals were sacrificed after 4, 6, 8, 10, and 24 weeks of diabetes. Gross examination of the arterial tree at 8 and 24 weeks of diabetes revealed an increase in the size as well as the number of lesions in the diabetic mice when compared to their age-matched, non-diabetic controls. Quantitation of atherosclerotic lesions at the aortic root revealed a 3.7-fold increase in mean lesion area in diabetic mice (143,967 ± 12,319 μm^2) compared with vehicle-treated controls (39,415 ± 9,875 μm^2) ($p<0.0001$). Total plasma cholesterol was 2.0-fold higher in diabetic mice ($p<0.05$), while total plasma triglycerides remained unchanged. Further analysis by density ultracentrifugation revealed a 1.4-fold increase in LDL cholesterol, and a 2.0-fold increase in the VLDL fraction, with relatively no change in HDL cholesterol. Increased AGE formation was confirmed by a 1.5-fold increase in AGE-reactive epitopes ($p<0.05$) as measured by ELISA using affinity-purified anti-AGE IgG in diabetic liver extracts. Immunohistochemistry of aortic lesions demonstrated markedly enhanced AGE-reactive epitopes in the vasculature of diabetic mice which co-localized with striking immunoreactivity for RAGE. Taken together, these data suggest an AGE-RAGE interaction in diabetic vessel wall may contribute to the pathogenesis of accelerated atherosclerosis in diabetes.

Acknowledgments

This thesis would not have been possible without the support and friendship of many people. I would like to first thank my advisor, Ann Marie Schmidt, for supervising and guiding this research. Her abundant enthusiasm, perpetual optimism, and dedication to her work have been a source of great inspiration to me. I will always be grateful to her for being my teacher, role model, and friend. I am also indebted to David Stern for playing an important role in my academic career, and to the Sarnoff Endowment for opening many doors of opportunity for me.

I have been fortunate to have worked with a number of terrific people in the laboratory, all of whom have contributed to this project in some way. My thanks to all of them, but especially to Kathy Raman, Kenneth Lee, June Wu, Evie Lalla, Yan Lu, June Li, and Luis Ferran, for their help and for putting a smile on my face every day.

I am also deeply grateful to my parents and my sister, Jeannie, who have supported me in all of my accomplishments over the years. Finally, I would like to express my gratitude to my husband, Dan, for his endless love and patience in seeing me through another thesis.

TABLE OF CONTENTS

INTRODUCTION

Heart disease was thought to be associated with diabetes as early as 1883, when Vergely recommended testing the urine of patients with angina for glucose [1]. Today we recognize cardiovascular disease to be the major cause of morbidity and mortality in people with diabetes mellitus. According to the 1987 National Hospital Discharge Survey, 77% of all hospitalizations for diabetic complications were for cardiovascular disease as compared to only 9% for renal disease, 6% for neurologic disease, 4% for eye disease, and 3% for other complications [2]. In addition, for every diabetic patient older than 20 years of age who died of renal disease, nine died of coronary heart disease and two died of stroke [3]. Overall, in North America about 75% of patients with diabetes ultimately die of cardiovascular causes [4].

Accelerated Atherosclerosis in Diabetes

Although ischemic heart disease is a leading cause of morbidity and mortality in the general population, a number of epidemiological studies have shown that individuals with diabetes have an increased incidence of cardiovascular disease [5, 6]. The prevalence of coronary artery disease is as high as 55% in adults with diabetes, compared to a 2 to 4% prevalence in the general population [7]. Angina is more common in persons with diabetes; the incidence is 60% higher in diabetic men and 90% higher in diabetic women than their non-diabetic counterparts [8]. Mortality statistics also underscore the impact of atherosclerosis on the diabetic population. Data from the Framingham Heart Study demonstrate that mortality from cardiovascular disease in non-insulin dependent diabetes

mellitus (NIDDM) is more than doubled in diabetic men and more than quadrupled in diabetic women when compared to their non-diabetic counterparts [9]. Additionally, results from the Joslin Study reveal that in patients with insulin dependent diabetes mellitus (IDDM), the mortality due to coronary artery disease is 35% by 55 years of age, far higher than the corresponding rate of 4-8% in persons without diabetes [9]. A particularly dramatic observation is the disproportionate impact of cardiovascular disease on pre-menopausal diabetic women compared with diabetic men, suggesting that this disease eliminates the well-known protection against ischemic heart disease normally experienced by women [10, 11].

Not only is atherosclerotic disease more prevalent in the diabetic population, but studies have shown that it is clearly accelerated and more extensive. In one large autopsy study, examination of coronary arteries from 23,000 individuals from 14 different countries demonstrated diabetes to be associated with an increase in the extent of atherosclerotic lesions regardless of the prevalence of coronary artery disease in the countries of origin [7]. Numerous other autopsy studies have revealed that patients with diabetes tend to have more severe disease of the left anterior descending artery [12], a higher incidence of two- and three-vessel disease [13], and a greater diffuseness of distribution of the atherosclerotic lesions [14]. Coronary angiographic studies in symptomatic patients have generally confirmed these findings [15].

Risk Factors for Atherosclerosis in Diabetes

These data indicate that diabetic individuals develop premature and more severe atherosclerotic disease than their non-diabetie counterparts. What accounts for this increased risk? One explanation is that diabetes is associated with a number of

established risk factors for cardiovascular disease. Those of particular importance include dyslipidemia, hypertension, and obesity which may predispose patients with diabetes mellitus to atherosclerosis and its complications.

Dyslipidemia

The term dyslipidemia refers to alterations in plasma concentrations of lipids, as well as abnormalities in the composition and metabolism of lipoproteins. The Framingham Heart Study revealed that the dyslipidemia of diabetes includes hypertriglyceridemia, low levels of high-density lipoprotein (HDL) cholesterol, and alterations in low-density lipoprotein (LDL), with a predominance of triglyceride-rich, small, dense LDL particles [16]. These changes which occur in both IDDM and NIDDM are exaggerated by poor metabolic control and may be modified after good glycemic control with diet and appropriate insulin therapy [17].

Hypertriglyceridemia, reflecting increased plasma levels of very-low-density lipoproteins (VLDL), is very common in both IDDM and NIDDM. While the role of triglyceride as a cardiovascular risk factor in non-diabetic individuals is debatable, studies of diabetics consistently and strongly suggest hypertriglyceridemia to be a significant risk factor for cardiovascular disease in this population [18]. Recent studies have shown that the size distribution of VLDL particles in this setting is shifted towards smaller, denser particles in the VLDL density range. While the reason that hypertriglyceridemia predisposes the diabetic individual to atherosclerosis is unknown, it has been hypothesized that these small VLDL particles may play a role [19].

While LDL has long been considered the most atherogenic circulating lipoprotein in non-diabetic individuals, the role of LDL in diabetic atherogenesis is less clear. Most

studies show that LDL levels are nearly normal or only mildly elevated in both types of diabetes [20]. These studies have shown, however, that the composition of the LDL particles is altered [21], with a higher prevalence of small, dense LDL particles which may be more atherogenic than normal LDL [22].

The circulating level of HDL is widely recognized to be a favorable predictor of coronary artery disease, with levels inversely related to the risk of developing atherosclerosis in the non-diabetic population. In patients with IDDM and NIDDM, levels of HDL cholesterol are uniformly low, and most studies show an association between low levels of HDL-cholesterol and increased risk of cardiovascular disease in this patient population [23].

Hypertension and Obesity

Hypertension is frequently associated with diabetes, with a prevalence twice as high in the diabetic population than in the non-diabetic population [24]. Although hypertension may result from diabetic nephropathy, the frequency of hypertension appears to be higher in the diabetic population even in the absence of renal disease [25].

The importance of abdominal obesity, as a risk factor for cardiovascular disease has long been appreciated. In particular, visceral adiposity confers a greater risk than does subcutaneous adiposity, since the former is associated with insulin resistance and compensatory hyperinsulinemia. Although this factor may not appear to be simple to detect clinically, recent studies have shown that this risk can be semiquantified by the waist-hip ratio [26].

Pathogenic Factors Unique to the Diabetic Population

Although all of these known cardiac risk factors likely contribute to the development of atherosclerotic vascular disease in diabetes, multivariate analysis indicates that diabetic patients have an excess risk of cardiovascular disease relative to non-diabetic subjects even after adjustment for conventional risk factors [27]. In the Multiple Risk Factor Intervention Trial (MRFIT), 350,000 men (5,245 with diabetes) were followed prospectively for 6 years. Even after accounting for major risk factors (serum cholesterol, blood pressure, smoking) the diabetic men had a three- to six-fold increased risk for cardiovascular disease. The Nurses Health Study followed 1500 diabetics among a total of 115,000 women, and found the incidence of cardiovascular disease to be five-fold higher in diabetics regardless of their cholesterol levels [28]. Thus, a large proportion of excess cardiovascular disease among diabetics cannot be explained by increases in known risk factors that affect the general population This observation suggests that there may be other atherogenic mechanisms which are unique to the diabetic population and play a critical role in the development of their disease.

Hyperglycemia as a risk factor

A number of epidemiological studies have strongly implicated hyperglycemia as an independent risk factor for cardiovascular disease in diabetes. The Tecumseh Study followed 921 men and 937 women over 40 years old who were without coronary artery disease upon entry into the study. After 12 years, diabetes was found to be a statistically significant independent risk factor for mortality due to coronary artery disease for both sexes. In addition, failure of a one-hour glucose challenge in patients not carrying the diagnosis of diabetes was also associated with excess mortality due to coronary artery

disease [29]. Similar findings were noted in the Chicago Heart Association Detection Project which compiled data on 11,230 men and 8,030 women who were followed prospectively for 9 years. In this study, both diabetes and asymptomatic hyperglycemia were associated with increased mortality from coronary artery disease [30].

One of the most compelling studies linking hyperglycemia with diabetic vascular complications is the Diabetes Control and Complications Trial (DCCT). The DCCT was a large, multicenter, controlled clinical trial designed to determine whether intensive treatment of hyperglycemia would affect the development and/or progression of complications in persons with IDDM. 1,441 patients were randomly assigned to conventional or intensive treatment with the goal of achieving glycemic levels as close to normal range as possible. Patients with hypertension, hypercholesterolemia, or obesity were excluded. The results showed that intensive therapy to decrease blood glucose concentrations to near normal levels effectively delayed the onset and slowed the progression of microvascular complications such as retinopathy, nephropathy, and neuropathy [31]. Additionally, the DCCT demonstrated that intensive treatment resulted in a significant reduction in cardiac risk factors such as mean total serum cholesterol, LDL cholesterol, and triglycerides. The number of combined major macrovascular events was almost twice as high in the conventionally treated group as in the intensive-treated group although the differences were not statistically significant [32].

Thus, the diabetic state in general and hyperglycemia in particular are associated with an increased risk for the development of cardiovascular disease. The mechanisms which may be involved in the pathogenesis of diabetic vascular disease will be discussed after a brief discussion of the basic mechanisms of atherosclerosis.

Mechanisms of Atherogenesis

Much knowledge has accumulated over the last few years concerning the basic mechanisms of atherogenesis. The development of atherosclerotic lesions occurs over many years with the appearance of fatty streak lesions which progress to fibrous plaques. As these atheromata increase in size, progressive pathological events ensue including calcification, ulceration, thrombus formation, and aneurysmal dilation. Restriction of blood supply to vital organs results in angina, myocardial infarction, and stroke, all clinical events seen with increased frequency in diabetes.

The earliest event that occurs during atherogenesis is the adhesion of monocytes to the endothelial lining of the vessel wall [33]. This interaction may be mediated by endothelial expression of mononuclear leukocyte adhesion molecule [34] or by integrins which are expressed on the surface of circulating leukocytes and bind to receptors on endothelial cells [35]. Therefore any factors that affect expression of adhesion molecules on either monocytes or endothelial cells may initiate fatty streak formation.

Once these monocytes penetrate the endothelial cells, they enter the subendothelial space and mature into macrophages [36]. Monocyte chemotactic protein-1 (MCP-1) is produced by macrophages and arterial smooth muscle cells and is believed to specifically attract monocytes to infiltrate the endothelial layer [37]. These monocytes are induced by various factors to differentiate into macrophages. While the identity of these agents is unknown, they are believed to be cytokines from T lymphocytes, which have been observed in close proximity to subendothelial macrophages [38]. Several cytokines have been implicated in the process of atherogenesis, including several interleukins, tumor necrosis factor, colony stimulating factor, fibroblast growth factor, vascular endothelial growth factor, platelet derived growth factor, and transforming growth factors α and β

[38]. The regulation and mechanism of these factors is largely unknown, but it is likely that any effect on the expression and activity of these cytokines may influence the development of atherosclerotic disease.

Once macrophages aggregate in the subendothelial space, they begin to accumulate lipids, predominantly cholesteryl esters, and develop into foam cells in the arterial wall. The lipids are derived from lipoproteins, leading to speculation on the mechanisms by which macrophages take up these lipoproteins. Much research has focused on the scavenger receptor on the surface of macrophages that bind oxidatively modified LDL [39, 40]. Macrophages also take up large amounts of lipid by phagocytosis of lipoprotein-immune complexes. Lipoproteins that have been modified by either oxidation or glycation can stimulate antibody production which presumably leads to the formation of lipoprotein-immune complexes which can be taken up by macrophages in the arterial wall. Additionally, lipoproteins may aggregate in the artery wall by binding to extracellular matrix components. These lipoprotein-glycosaminoglycan complexes can also be taken up by macrophages, leading to foam cell formation [41].

While not all fatty streak lesions are thought to progress to complicated atherosclerotic plaques, it is believed that all complicated lesions originate from fatty streaks. Lesions that progress to fibro-fatty plaques are characterized by smooth muscle cell migration from the media to the intima. These cells proliferate and synthesize connective tissue which is deposited in the vessel wall, leading to fibrous cap formation. The proliferation and activity of these smooth muscle cells is also likely under the influence of a number of growth factors, such as platelet derived growth factor [42], whose regulation may influence the development of this disease.

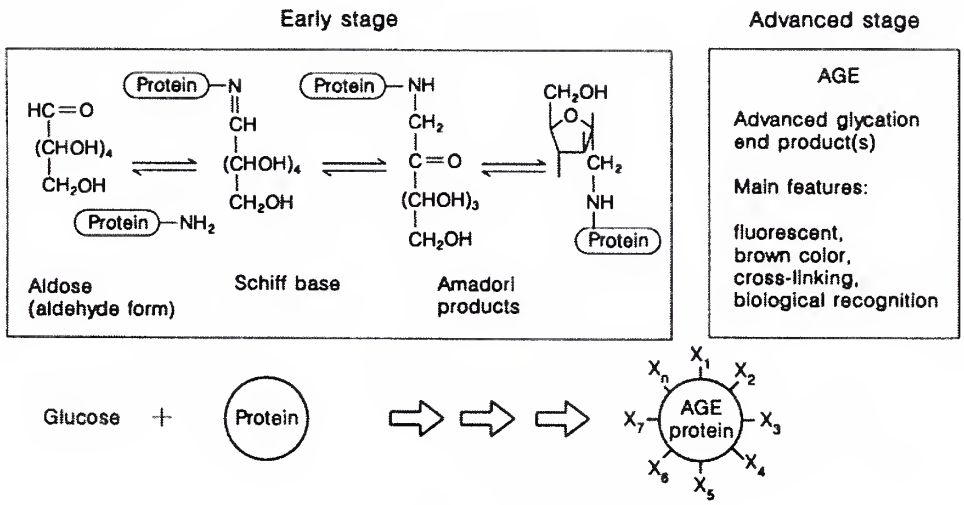
Atherogenesis in Diabetes

The reason for the accelerated development of atherosclerosis in diabetes is not known. As discussed earlier, conventional risk factors are insufficient to explain the tremendous incidence of cardiovascular disease in diabetes, raising the likelihood that atherogenic mechanisms unique to the diabetic population must be involved. Under conditions of sustained hyperglycemia, proteins and lipids are modified by nonenzymatic glycation and oxidation, and we must consider the possibility that the products of these reactions may play a role in the development of diabetic vascular disease.

Formation of Advanced Glycation Endproducts

In diabetes, one of the central alterations in homeostasis is the nonenzymatic glycation of proteins and lipids leading initially to the formation of early reversible complexes and resulting ultimately in stable advanced glycation endproducts known as AGEs. [43, 44]. This process begins with the condensation of a sugar aldehyde or ketone with the reactive amino group of an amino acid residue (usually lysine) forming a Schiff base. Equilibrium is reached in a matter of hours at a steady-state level that is proportional to the glucose concentration. The Schiff base undergoes rearrangement over a period of days to weeks to form the more stable Amadori product, a 1-amino-1-deoxyketose compound. The Amadori product is then degraded into a variety of highly reactive carbonyl compounds such as 3-deoxyglucosone and sugar fragmentation products which react again with protein amino groups in a complex series of rearrangements to form a variety of intermediate and stable endproducts. These advanced glycation endproducts, or AGEs, undergo a rate of formation that is approximately second order with respect to the concentration of glycated amino groups, suggesting that

even modest elevations of glucose significantly increase AGE formation. In addition, because AGEs are the products of an irreversible reaction, the level of AGEs does not decline when hyperglycemia is corrected. Instead, these molecules continue to accumulate over the lifetime of the diabetic tissue component [45].



AGEs arise from a complex series of dehydrations, rearrangements, and reactions that are poorly understood [46]. However, this heterogeneous class of compounds share a number of characteristics which include yellow-brown color, characteristic fluorescence, tendency to form cross-links, ability to generate reactive oxygen intermediates, and the ability to interact with specific cellular receptors. Thus far, only two AGE products have had their structures fully characterized. One is carboxymethyllysine [47], which is formed when oxygen free radicals cleave fructose-lysine residues. The other, pentosidine [48], forms when glucose moieties from two glycated amino acids (1 lysine, 1 arginine) link together. While it is unclear whether either of these two molecules have any clinical relevance, they may serve as markers for other products which may be directly involved

in the pathogenesis of disease states. In terms of how glycation products may play a role in atherogenesis in the setting of diabetes, much research has focused on AGE modification of two particular species: serum lipoproteins (primarily LDL) and structural proteins (primarily collagen).

AGE modification of serum lipoproteins

One known target of glycation is apolipoprotein B on the surface of LDL. Glycation of LDL under hyperglycemic conditions was first demonstrated more than 10 years ago by in vitro experiments which showed that the extent of LDL correlated with the duration of incubation and the concentration of glucose in the incubation mixture. In vivo glycation of apolipoprotein B was also found to be increased in diabetic patients, leading to the hypothesis that glycated LDL contributes to the accelerated atherosclerosis of diabetes [49].

A number of mechanisms involving glycated LDL have been investigated. Studies have shown that recognition of glycated LDL by the classic LDL receptor is impaired, increasing its half-life in plasma and its exposure to oxygen free radicals [50]. The increased susceptibility of LDL to subsequent oxidative damage may enhance its uptake by subendothelial macrophages, stimulating foam cells formation [51]. Additionally, glycation of LDL may increase glucose-mediated covalent binding and sequestration within vessel walls, particularly if the matrix structural proteins have themselves been modified by glycation. LDL modification may also provoke an antibody response with the formation of potentially atherogenic LDL-immune complexes [52].

AGE modification of extracellular matrix

Because insoluble collagen is a long-lived protein existing in the matrix for years, it is a likely target for modification by glycation mechanisms. Studies have shown that in normal subjects, glycation of collagen in skin increases only slightly with age [47]. Diabetic patients, however, exhibit a substantial increase in collagen glycation which correlates with levels of HbA1c, a marker of long-term glycemic control [53].

The physical changes which occur upon glycation of collagen include the formation of covalent, heat-stable, intermolecular bonds [54]. The cross-links derived from AGE formation may be pertinent to atherogenesis for a number of reasons. The increased crosslinking of collagen may cause abnormal vascular rigidity and tone contributing to hypertension and as well as hyperpermeability and abnormal shear stresses involved in endothelial injury. Additionally, replication of vascular cells such as endothelial cells and smooth muscle cells, has been shown to be inhibited in the presence of glycated collagen matrix, thereby preventing repair processes that require the proliferation of these cells [55]. The glycation of collagen may also alter normal interactions of matrix ligands with plasma constituents. For example, it may cause plasma constituents such as lipoproteins and immune complexes to be more adherent to the vessel wall [56]. In addition, AGE modified matrix has been shown to be chemotactic to monocytes, facilitating their migration into the vessel wall, differentiation into macrophages, and formation of foam cells. This interaction, as well as a number of others, has been postulated to be mediated by specific cellular receptors that bind to a common structural element in AGEs.

Receptors for AGEs

The first receptor for AGEs was identified on monocytes and macrophages [57]. This discovery was of unique significance because it is the first receptor that recognizes a posttranslational protein modification known to occur extensively in vivo. Binding of AGEs to this receptor induces macrophage production of interleukin-1 and insulin-like growth factor 1, in addition to tumor necrosis factor α. These induced cytokines have been shown to activate proliferation of arterial smooth muscle cells as well as stimulate proliferation of glomerular mesangial cells and glomerular synthesis of collagen [58, 59].

AGE-specific receptors have also been found to be expressed on the surface of endothelial cells [60]. Initial studies showed that ligand binding to this receptor induces procoagulatory changes by increasing tissue factor activity and reducing thrombomodulin activity, resulting in vasoconstriction and focal thrombosis. This receptor, named RAGE (receptor for advanced glycosylation end products), was subsequently cloned and characterized [61]. This molecule is a member of the immunoglobulin superfamily of cell surface molecules, whose extracellular region consists of one variable and two constant domains. Following this region is a single transmembrane-spanning region and short, highly charged cytosolic tail. Thus, RAGE is an integral membrane protein capable of serving as a cell-surface acceptor site for AGEs.

In situ studies of endothelial RAGE using AGEs tagged with colloidal gold have demonstrated that after surface binding, AGEs are endocytosed and a portion of the ligand traverses the endothelial cell layer by transcytosis. Thus, RAGE provides a pathway for receptor-mediated uptake of ligands from the intravascular space with delivery to the subendothelium where AGEs may interact with extracellular matrix components [62]. This receptor is also expressed on the surface of mononuclear

phagocytes, and in vitro chemotaxis studies indicate that this receptor is able to mediate monocyte migration in response to AGEs [63]. This interaction may provide a mechanism for attracting and retaining macrophages at sites of AGE deposition in tissues and contribute to the development and progression of atheroclerotic lesions.

Recent studies have also implicated the involvement of AGE-RAGE interaction in the endothelial expression of VCAM-I, which has been associated with early phases of atherosclerosis [64, 65]. In vitro studies of human endothelial cells exposed to AGEs showed increased expression of VCAM-I as measured by ELISA and mRNA studies, and this effect was blocked by antibodies to RAGE. In vivo studies in which AGE-albumin was infused into normal mice also showed enhanced expression of VCAM-I in the vasculature by immunohistochemical analysis [66]. These data suggest a unique pathway by which AGEs may contribute to the pathogenesis of diabetic vascular disease.

Animal Models of Diabetes

Our understanding of diabetes mellitus has benefited greatly by the use of animal models of disease. The use of animals, rather than humans, in diabetes research has multiple advantages. In studying the pathogenesis of this disease, animal models permit more complete analysis of anatomical and biochemical changes in organs which are generally inaccessible in humans. Use of a homogeneous population maintained under well-controlled conditions also allows for the isolation of one of many different factors which may influence the development of disease in humans. Additionally, animal models provide us with the opportunity to investigate the toxicity and efficacy of therapeutic measures to prevent the development or progression of the disease and its complications.

A number of animals have been used to study diabetes. These include non-human primates, dogs, swine, and other large animals in which diabetes can be induced using viruses, chemical agents, or by partial pancreatectomy. However, these animal models pose many technical difficulties and high maintenance costs in cultivating large colonies necessary for statistically significant sample sizes. These animals also do not allow for genetic manipulation in order to dissect out the underlying biochemical and molecular causes for this disease. For this reason, the rodent has been an often used model which has been well-characterized. Some of the more commonly used genetic mouse models of diabetes include the autosomal recessive db/db mouse and the NOD (non-obese diabetic) mouse. Their use in the specific study of diabetic vascular disease has been limited, however, by the inherent resistance of mice to the development of atherosclerotic disease [67].

Recently, a transgenic mouse has been developed, the apolipoprotein E deficient mouse, which develops advanced atherosclerosis on a normal chow diet [68, 69]. This animal develops hypercholesterolemia primarily due to elevated levels of very low and intermediate density lipoproteins, and demonstrates a full range of atherosclerotic lesions from fatty streaks to fibrous plaques. These lesions are consistently distributed throughout the arterial tree in sites of known predilection. Thus, by using chemical agents to induce diabetes, this mouse may provide us with a useful animal model to study mechanisms of accelerated atherosclerosis in diabetes.

STATEMENT OF PURPOSE

My objective is to develop and describe an animal model of accelerated atherosclerosis by inducing diabetes in apolipoprotein E deficient mice. We believe this model will provide an important means to study the lipid-dependent and lipid-independent mechanisms which underlie accelerated atherosclerosis in diabetes. We also hope to use this model to test our hypothesis that the formation and accumulation of AGEs under sustained hyperglycemia, and their interaction with the cellular receptor, RAGE, contribute to the pathogenesis of this disease.

MATERIALS AND METHODS

Preparation and staining of aortic tissue sections were performed with the technical assistance of Yan Lu and Yu Shan Zou. All other methods described below were performed by Lisa Park.

Animals

Apolipoprotein E deficient mice on a C57Bl/6J background (N10 generation, 99% homology) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in a barrier facility with controlled temperature (65-75° F) and humidity (40-60%). The mice were maintained on a 12 hr light/dark cycle and given free access to food and water. The animals were maintained on a normal rodent chow diet (Purina 5001) for the duration of the experiments. Diabetes was induced in male apolipoprotein E deficient mice at 7 weeks of age, using multiple intraperitoneal injections of streptozotocin (STZ, Sigma) dissolved in a sterile citrate buffer (pH 4.5). STZ (55 mg/kg) or vehicle alone was administered for 6 days. Plasma glucose concentration was determined by colorimetric assay (Sigma) using blood obtained from the tail vein. Mice were considered diabetic if glucose levels were greater than 300 mg/dl on two separate occasions. Glycosylated hemoglobin was measured from packed red cells by affinity chromatography (Pierce). Mice were euthanized after 4, 6, 8, 10, and 24 weeks of diabetes using an intraperitoneal injection of ketamine and xylazine. Blood was drawn through a 30 gauge needle via the inferior vena cava. The liver and kidneys were resected and frozen immediately at -80°C. A thoracotomy was performed and the beating heart and arterial tree were perfused with

phosphate-buffered saline (PBS, GIBCO) via the inferior vena cava. The heart and aorta were dissected away from the thorax and stored immediately in formalin or frozen at -80°C until processed for further analysis.

Quantitation of atherosclerotic lesions

Quantitative analysis of atherosclerotic lesions was performed on sections from the aortic sinus. The hearts were fixed in 10% formalin, embedded in 25% gelatin and frozen. Cryostat sections were cut 10 microns thick throughout the aortic sinus, stained with oil red O and counterstained with hematoxylin and light green. For each animal, fatty lesion areas were measured by computer assisted image analysis (Zeiss Image, Media Cybernetics) in five consecutive sections each separated by 80 microns. The mean lesion area per section per animal was calculated for each group of mice.

Lipoprotein analysis

Mice were fasted for four hours prior to obtaining plasma for lipoprotein analysis. Blood was collected by retro-orbital puncture in 0.005M EDTA, and plasma concentrations of cholesterol and triglyceride were measured using commercial kits (Boehinger Mannheim). VLDL ($d < 1.006$ g/ml), IDL/LDL ($d = 1.006$-1.063 g/ml), and HDL ($d=1.063$-1.21 g/ml) were separated by sequential density ultracentrifugation of pooled plasma. Subfractionation of the VLDL fraction was performed by overlaying with nonlinear salt gradients and removing the top layer after three successive centrifugations. Levels of cholesterol and triglyceride were measured in each of these fractions.

AGE ELISA

Frozen mouse livers were homogenized and incubated in a non-ionic detergent, 1% n-octylglucoside (Boehinger Mannheim), at 4°C overnight. After ultracentrifugation, the supernatant was collected and protein concentration determined by the Bradford assay (Biorad). ELISA was performed on these samples using chicken anti-AGE IgG primary antibody. The secondary antibody was a peroxidase-conjugated rabbit anti-chicken IgG (Sigma). Spectrophotometric readings were taken using o-phenlenediamene dihydrochloride (OPD, Sigma) as the chromagen.

Immunohistochemistry for AGE and RAGE

Aortic tissue was fixed in 10% formalin; cross-sectional and longitudinal sections were prepared. Immunohistochemistry for AGE was performed using chicken anti-AGE IgG and immunohistochemistry for RAGE was performed using rabbit anti-RAGE IgG. Peroxidase-conjugated rabbit anti-chicken IgG and goat anti-rabbit IgG (Sigma) were used as secondary antibodies. Non-immune rabbit and goat serum were used as negative controls.

RESULTS

1. Hyperglycemia is induced in apolipoprotein E deficient mice treated with streptozotocin.

Our goal was to investigate the role of hyperglycemia on the development of atherosclerotic disease without the confounding factor of concurrent insulin administration to maintain animal viability. Therefore, we used a multiple low-dose injection regimen of streptozotocin and documented hyperglycemia on day 10 following the start of treatment. These streptozotocin-treated mice were relatively healthy as evidenced by adequate coat color, activity, weight gain, and absence of ketonuria. Plasma glucose levels measured during the course of the experiments ranged from ~350-500 mg/dl for the STZ-treated mice as compared with ~130-160 mg/dl for the citrate-injected controls (see Figure 1). Overall, these levels were nearly tripled for the mice injected with streptozotocin as compared with their citrate-treated controls.

Glycosylated hemoglobin, or HbA_{1c}, is an early glycation product which reflects average blood glucose levels over a two to three month period. Measurements were performed by affinity chromatography on packed red blood cells. Mean levels of HbA_{1c} were 7.18 ± 1.7% in the diabetic mice compared to 4.03 ± 1.2% for their age-matched controls (see Figure 2). Thus, diabetic mice demonstrated a 1.8-fold higher ($p<0.05$) glycosylated hemoglobin over their controls, confirming a state of sustained hyperglycemia.

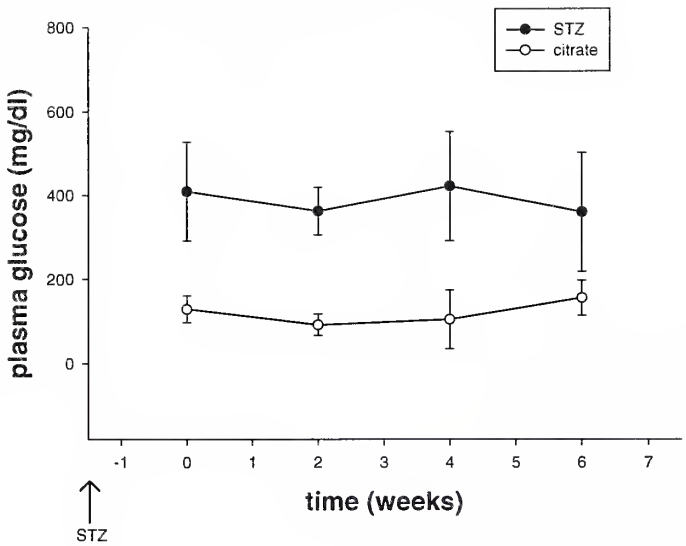


Figure 1 **Plasma glucose concentrations of apolipoprotein E deficient mice after treatment with streptozotocin or citrate.** Animals were treated with intraperitoneal injections of STZ (55 mg/kg) or citrate buffer for 6 days. Plasma glucose concentration was determined by colorimetric assay on blood obtained from the tail vein. Glucose levels were nearly tripled for the streptozotocin-treated mice compared to the citrate-treated controls.

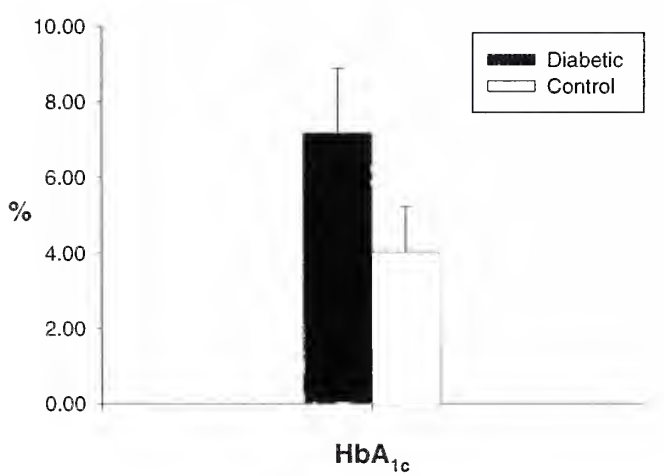


Figure 2 **Glycosylated hemoglobin levels after treatment with streptozotocin or citrate.** Levels of HbA1c were determined by affinity chromatography on packed red blood cells obtained 8 weeks after treatment with STZ or citrate. Mean levels were 1.8-fold higher in the diabetic animals compared to the non-diabetic controls ($p<0.05$).

2. *Diabetic mice demonstrate accelerated atherosclerosis.*

It has been previously described that the apolipoprotein E deficient mouse develops atherosclerotic lesions on a normal rodent chow diet. These lesions develop throughout the aorta and its principal branches at known sites of predilection (see Figure 3). Gross lesions first appear as small yellowish-white nodules detectable at 10 weeks of age which increase in size and progress in appearance to typical fibrous plaques at approximately 20 weeks of age.

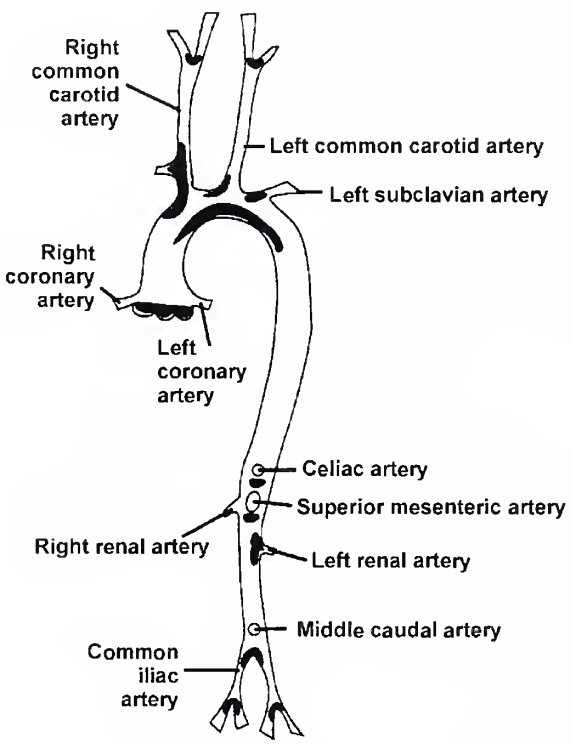


Figure 3 **Sites of predilection for lesion development in the apolipoprotein E deficient mouse** (reprinted with modification from Nakashima, 1994). These include the aortic root, lesser curvature of the aortic arch, principal branches of the thoracic aorta, carotid artery, principal branches of the abdominal aorta, aortic bifurcation and iliac artery.

By inducing diabetes in these mice, we investigated whether the development of atherosclerosis would be accelerated in this animal model. Mice were dissected at 4, 6, 8, 10, and 24 weeks of diabetes. Fatty streak lesions were observed in both diabetic and control mice, as expected. These generally appeared first at the aortic root and at the lesser curvature of the arch, with progression to each of the principal branches of the thoracic aorta, beginning proximally. At each timepoint examined, the lesions were consistently larger in size and more extensive in the diabetic animals when compared to their age-matched controls. For example, after 10 weeks of hyperglycemia, the diabetic mouse had discrete lesions at each of the thoracic branchpoints with nearly complete occlusion of the vessels (see Figure 4). This is in marked contrast to an age-matched control mouse which had some mild fatty streaks, mainly at the aortic root, which were difficult to appreciate grossly.

With increasing age, atherosclerotic lesions progressed throughout the aorta and its principal branches. After 24 weeks of diabetes, the STZ-treated mice demonstrated a marked increase in the size as well as the number of lesions over their age-matched controls throughout the arterial tree, particularly in the descending thoracic aorta, the renal artery branches, and at the iliac bifurcation (see Figure 5). The appearance of these lesions was typical of fibrous plaques with substantial narrowing of the arterial lumen, in some cases to the point of ~95% occlusion.

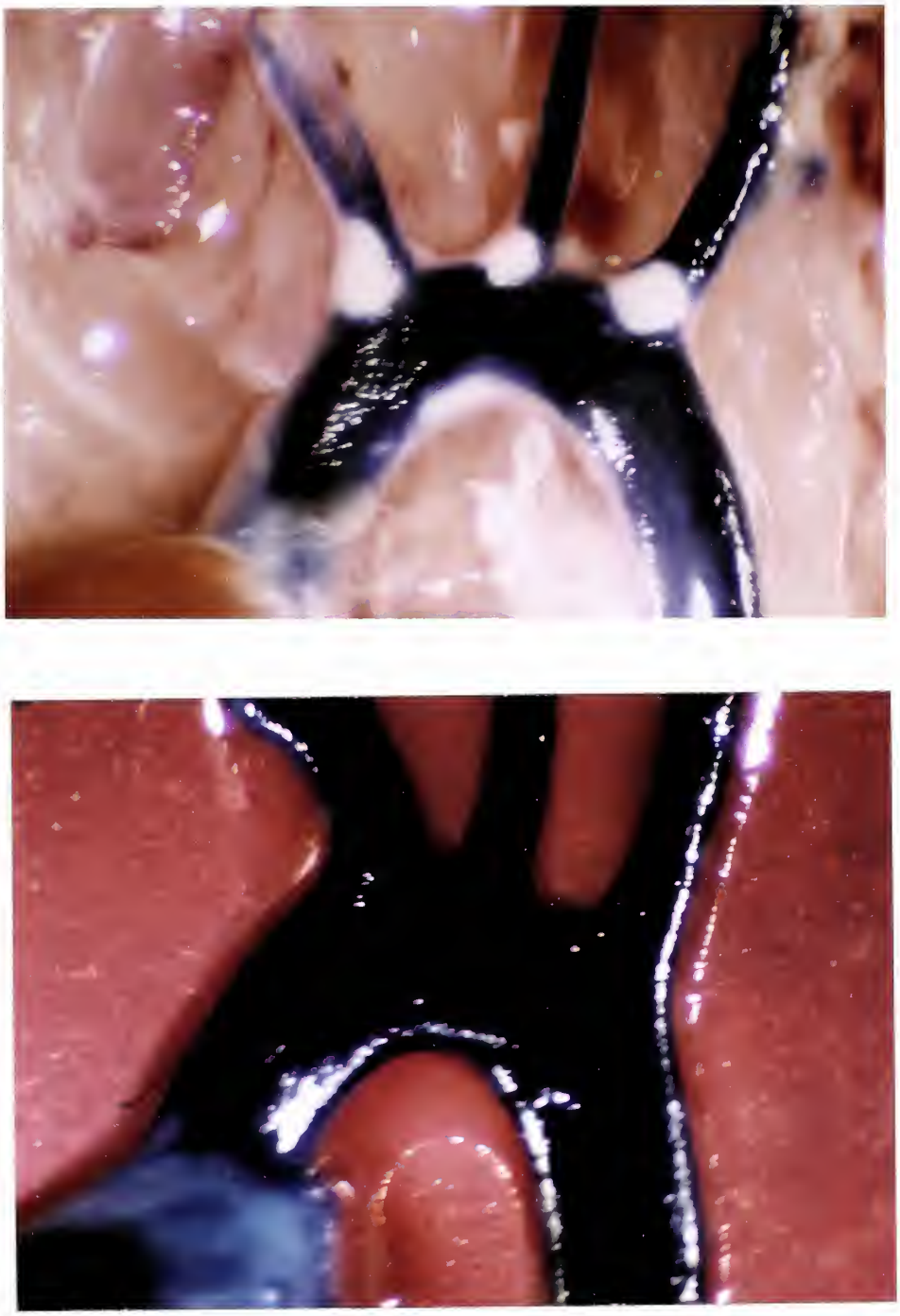


Figure 4 **Gross examination of the proximal aorta under dissection microscopy.** (4x magnification) Aortic specimens were treated with a retrograde injection of methylene blue to highlight the white atherosclerotic lesions developing within the aortic lumen. Diabetic (top) and control (bottom) mice were 16 weeks of age (10 weeks of diabetes).

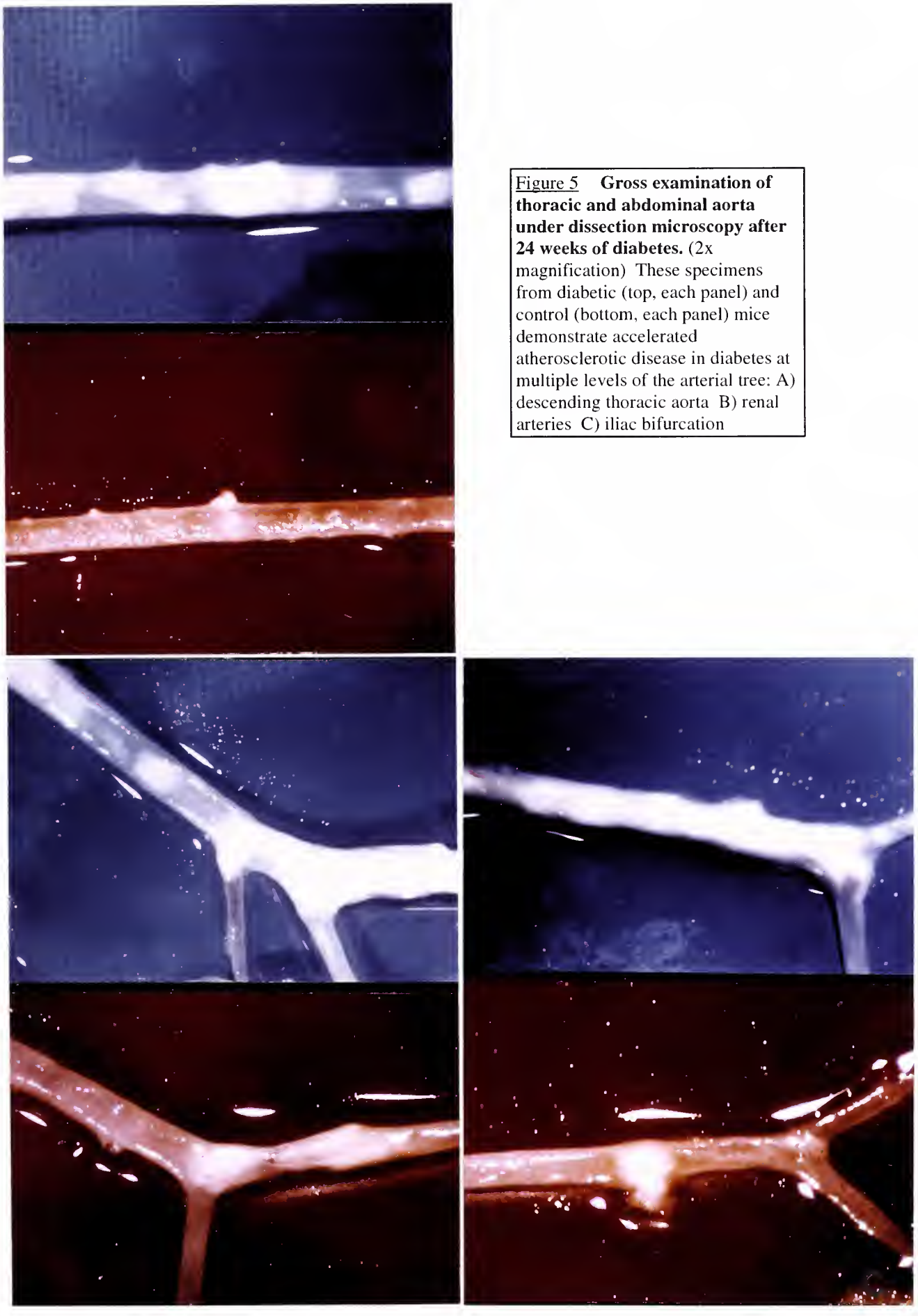


Figure 5 **Gross examination of thoracic and abdominal aorta under dissection microscopy after 24 weeks of diabetes.** (2x magnification) These specimens from diabetic (top, each panel) and control (bottom, each panel) mice demonstrate accelerated atherosclerotic disease in diabetes at multiple levels of the arterial tree: A) descending thoracic aorta B) renal arteries C) iliac bifurcation

In order to quantitate the degree of atherosclerosis for comparison between the diabetic and control groups, histological specimens from the aortic root were obtained from the mice in a standardized manner. Cryostat sections were cut specifically at the aortic sinus beginning at the point of vessel rounding once the three valves were visualized. These were stained using oil-red-O, a lipid-specific stain, and counterstained with hematoxylin and light green.

Light microscopic examination of aortic tissue sections from all of the mice demonstrated fatty lesions. In the non-diabetic mice, the vascular lesions were characterized generally by small groups of lipid-filled cells found primarily adjacent to valve attachment sites. In contrast, specimens taken from diabetic mice after only 6 weeks of hyperglycemia demonstrated more advanced atherosclerotic lesion formation. These consisted of foam cells intermixed with spindle shaped smooth muscle cells tending to form a fibrous cap on top of the lesion. (see Figure 6)

Quantitation of the fatty streak lesion areas was performed in 5 consecutive sections each separated by 80 microns, and the mean lesion area per section was calculated using computer-assisted image analysis (see Figure 7). The mean lesion area in the control group of 16 week old apolipoprotein E deficient mice was 39,415 ± 9875 μm^2. In comparison, at the same age, but after 8 weeks of sustained hyperglycemia, the mean lesion area was 143,967 ± 12,319 μm^2; a statistically-significant 3.7-fold increase over the non-diabetic controls ($p<0.0001$). A similar experiment after 6 weeks of diabetes revealed a 3-fold increase ($p<0.05$) in the size of diabetic atherosclerotic lesions.

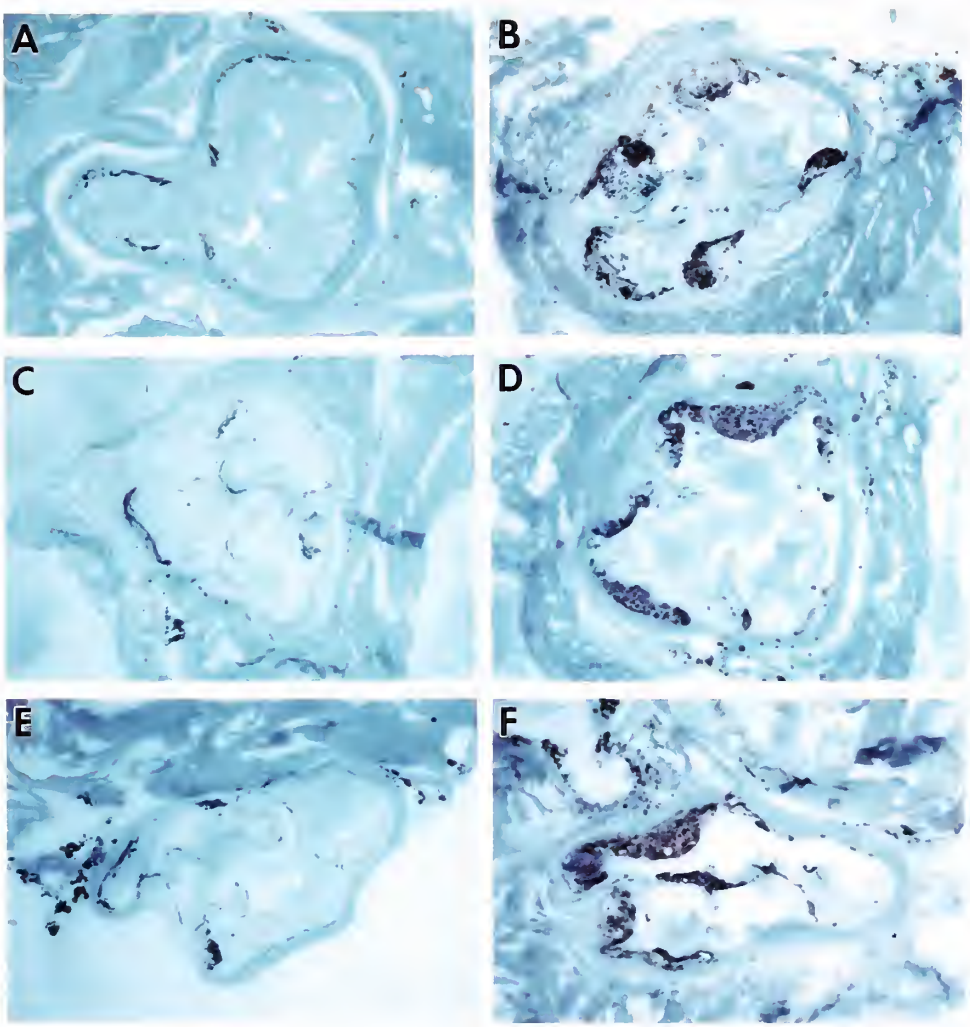


Figure 6 **Oil red O-stained aortic sections from diabetic and control apolipoprotein E deficient mice fed a normal chow diet.** (40x magnification) Aortic sections were stained with oil red O and counterstained with hematoxylin and light green. Diabetic mice (B,D,F) demonstrated marked accelerated atherosclerotic lesion development after 8 weeks compared to the non-diabetic age-matched controls (A,C,E).

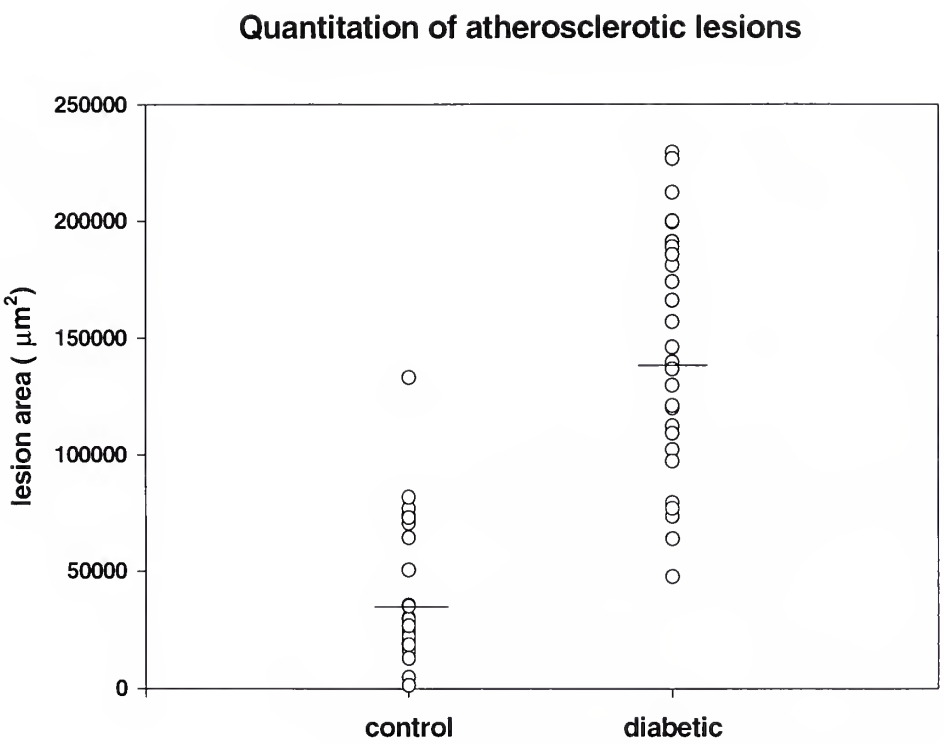


Figure 7 **Aortic sinus lesion areas for diabetic and control apolipoprotein E deficient mice**. Quantitation of atherosclerotic lesions was performed using computer-assisted image analysis (ImagePro, Media Cybernetics). Mean lesion area for diabetic mice (143,967 ± 12,319 μm^2) was 3.7-fold higher compared to age-matched controls (39,415 ± 9875 μm^2) after 8 weeks of diabetes ($p<0.0001$)

3. Lipid analysis

To document the level of plasma lipids over time, mice were fasted for four hours prior to sacrifice at various timepoints. Blood was obtained via the inferior vena cava, and total plasma cholesterol and triglyceride levels were determined by colorimetric assay. Plasma cholesterol levels of the diabetic mice were approximately double that of the control mice after 4 weeks of diabetes, while the triglyceride levels remained unchanged. The cholesterol levels of the diabetic mice after 8 weeks of diabetes ranged from ~650-1800 mg/dl with a mean of 1244 mg/dl whereas those of the age-matched controls ranged from ~450-630 mg/dl with a mean of 543 mg/dl. There was greater variability in the cholesterol levels of the diabetic animals, with levels that were consistently higher than those of the non-diabetic controls (see Figure 8).

In order to determine the distribution of cholesterol among the different lipoprotein fractions, density ultracentrifugation was performed on pooled plasma (see Figure 9). This analysis revealed that the greatest cholesterol increase occurred in the VLDL fraction, which showed a 2.0-fold increase in the diabetic mice over their age-matched non-diabetic controls. There was also a 1.4-fold increase in the LDL cholesterol level. HDL cholesterol remained relatively unchanged between the two groups.

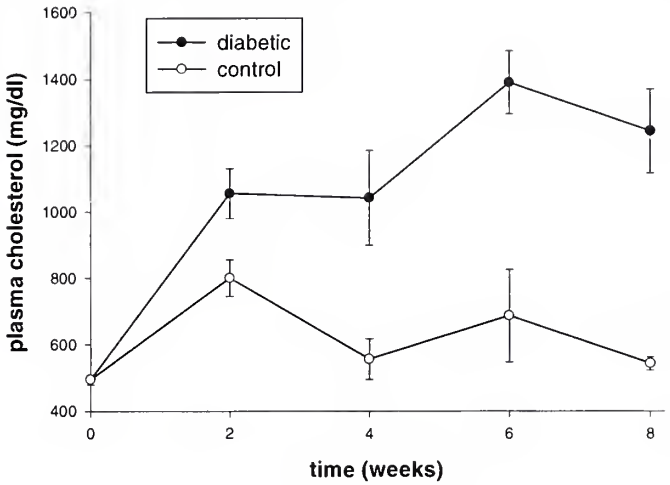


Figure 8 **Plasma cholesterol levels of diabetic and control mice.** Plasma cholesterol concentrations after a 4 hour fast were determined by colorimetric assay. At 4, 6, and 8 weeks of diabetes, the cholesterol levels of the diabetic mice were two-fold higher than the age-matched controls.

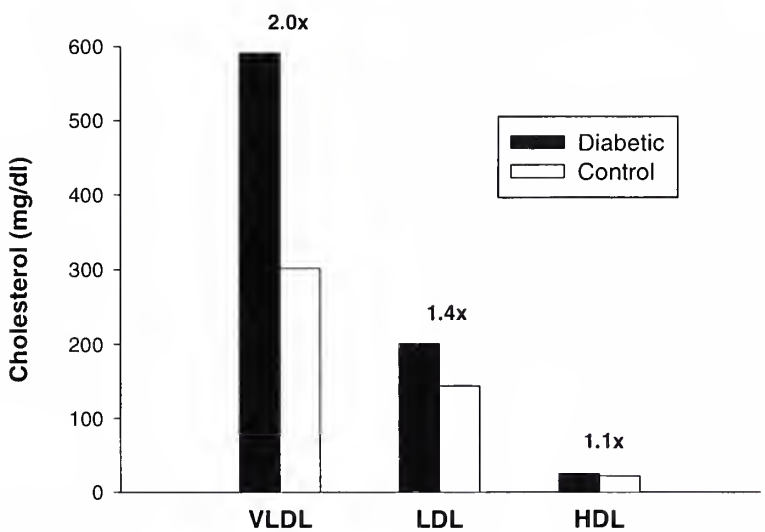


Figure 9 **Cholesterol concentrations of lipoprotein fractions.** Lipoprotein fractions were separated by density ultracentrifugation of pooled plasma. The greatest increase was in the VLDL fraction, which was 2.0-fold higher in the diabetic mice over the non-diabetic controls. LDL cholesterol increased 1.4-fold, while HDL cholesterol was relatively unchanged.

4. AGE formation is enhanced in these diabetic animals.

AGEs were measured directly in protein extracts from mouse liver by ELISA, using affinity-purified anti-AGE IgG antibody developed in our laboratory. Mean AGE levels in the diabetic mice were 144 ± 26 µg/dl as compared with 98 ± 17 µg/dl in the age-matched controls. Thus, diabetic mice demonstrated a 1.5-fold ($p<0.05$) increase in AGEs over their age-matched, non-diabetic controls as measured in this highly vascularized organ (see Figure 10).

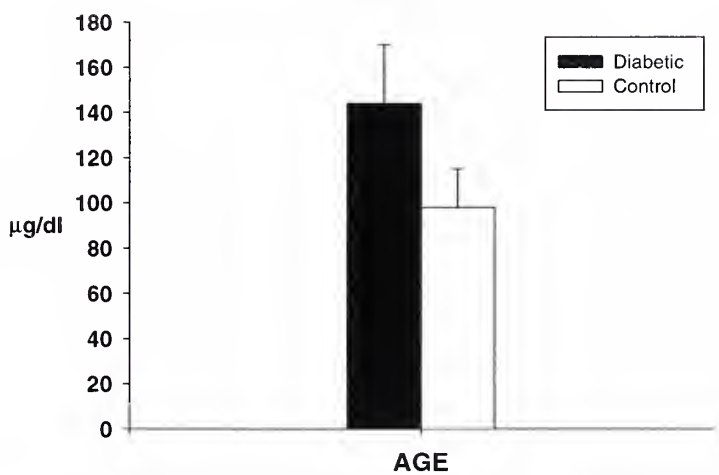


Figure 10 **Evidence of elevated AGEs in diabetic mice compared with non-diabetic controls.** Levels of AGEs were measured directly by ELISA using affinity-purified anti-AGE IgG in mouse liver extracts. Diabetic mice showed a 1.5-fold increase ($p<0.05$) in AGEs compared to non-diabetic animals.

5. AGEs accumulate in the vasculature of diabetic mice.

In order to investigate the possible role of AGEs in the development of accelerated atherosclerosis, we sought to identify AGEs within the vasculature. We performed immunohistochemical analysis of aortic tissue sections after 6 weeks of hyperglycemia. The diabetic mouse shows increased staining for AGE reactive epitopes in the vessel wall, with the highest levels deposited in the endothelium of the tunica intima as well as in the collagenous fibers of the tunica adventitia (see Figure 11).

6. RAGE expression is enhanced in the diabetic vessel wall.

Previous studies have shown that the receptor for AGEs, RAGE, is expressed by endothelial cells as well as smooth muscle cells in the vasculature. We investigated the expression of RAGE in our mouse model by immunohistochemical analysis of aortic sections using polyclonal monospecific anti-RAGE IgG. There is enhanced expression of RAGE in the vessel wall of the diabetic mouse as compared to its non-diabetic control. This staining is prominent throughout the endothelium and the smooth muscle cells of the tunica media after only 6 weeks of diabetes (see Figure 12).

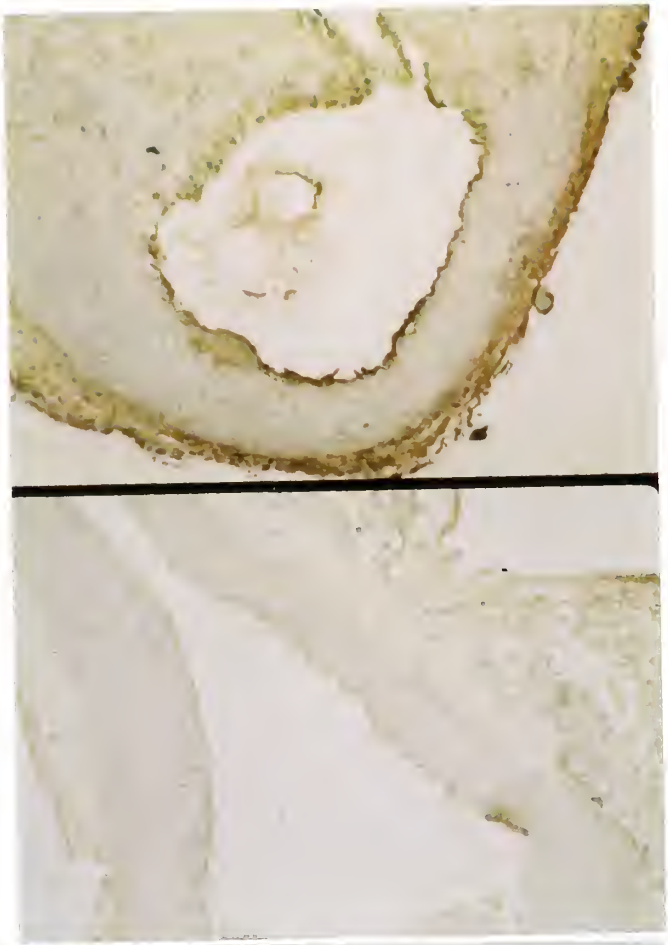


Figure 11 **Immunohistochemical staining for AGE in diabetic (top) and control (bottom) mouse aorta.** (40x magnification) Tissue sections were stained with polyclonal anti-AGE IgG. Aortic sections from diabetic mice demonstrate a marked increase in AGE-reactive epitopes in the tunica intima and tunica adventitia when compared to non-diabetic control mice.

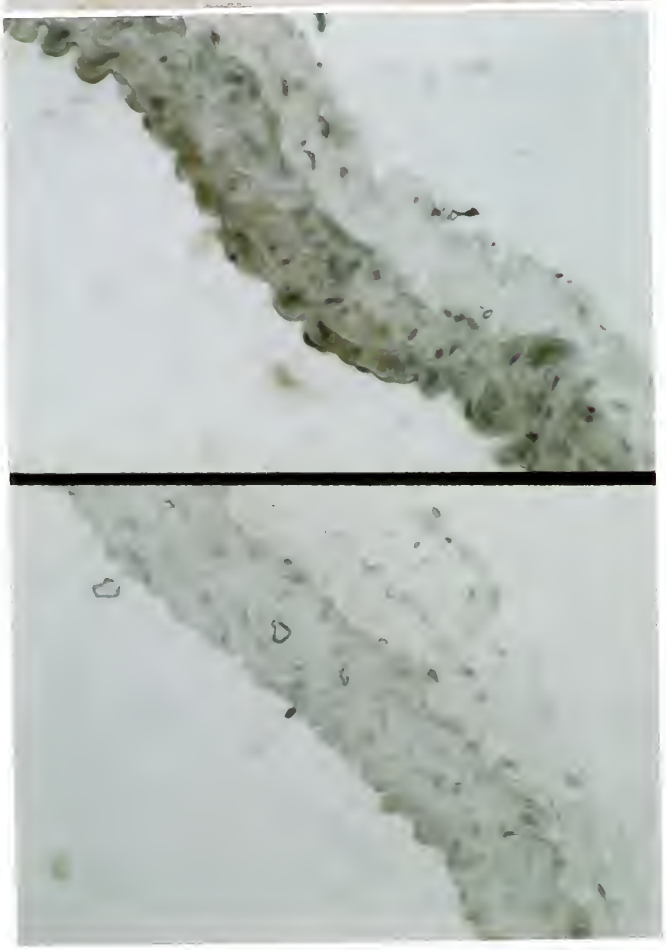


Figure 12 **Immunohistochemical staining for RAGE in diabetic (top) and control (bottom) mouse aorta.** (100x magnification) Tissue sections were stained with polyclonal anti-RAGE IgG. Diabetic mice demonstrated marked RAGE expression in the endothelial and smooth muscle cells of the vasculature compared to the non-diabetic controls.

DISCUSSION

Development of an animal model of accelerated atherosclerosis

One of the primary objectives of this study was to develop a mouse model of accelerated atherosclerosis in diabetes. By using a multiple low-dose injection regimen of streptozotocin, we were able to achieve sustained hyperglycemia and animal viability in the apolipoprotein E deficient mouse. Because these animals were maintained without concurrent insulin administration, we were also able to avoid the introduction of hyperinsulinemia as an independent risk factor for cardiovascular disease.

The diabetic mice exhibited significantly accelerated atherosclerosis when compared to their age-matched, non-diabetic counterparts at multiple timepoints. The initial lesions appeared earlier in the diabetic animals with a significant increase in size observed upon gross examination of the aorta after only 6 weeks of diabetes. Quantitation of fatty streaks at the aortic root demonstrated a nearly four-fold increase in the size of the lesions by 8 weeks of diabetes, and by 24 weeks there was extensive atherosclerotic disease throughout the arterial tree with nearly 100% occlusion of major branches.

Not only were the atherosclerotic lesions larger in size, but the disease was also more extensive and advanced in the diabetic animals. Histologic examination revealed foam cell and fibrous cap formation in aortic sections after 8 weeks of diabetes, while the non-diabetic animals demonstrated early fatty streak lesions at the same age. Thus, by chemically inducing diabetes in this mouse, we have created an experimental model of accelerated atherosclerosis.

By using the apoliprotein E deficient mouse which has a natural propensity to develop atherosclerosis, we eliminated the need to administer an atherogenic diet. Previous studies have attempted to overcome the inherent atherosclerosis resistance of mice by feeding a diet consisting of 1.25% cholesterol, 15% saturated fat, and 0.5% cholic acid [70]. This diet contains 10-20 times the amount of cholesterol found in a typical human diet and contains an unnatural dietary substance, cholic acid. When fed this cholesterol-rich food, certain inbred mouse strains, such as C57BL/6J, will show atherosclerotic changes [71]. However, the lesions that do eventually develop are relatively immature in quality and restricted in distribution to the aortic arch. Another mouse strain, BALB/c, has also been shown to develop atherosclerosis while fed an atherogenic diet [72]. When this strain is treated with STZ, accelerated atherosclerosis does result. However, the diabetic mice on normal chow develop no vascular disease, suggesting that both dietary as well as diabetic factors act in combination in this model. By eliminating the need for an atherogenic diet, the apolipoprotein E deficient mouse allows us to isolate the effects of hyperglycemia alone.

Lipid analysis of the diabetic apolipoprotein E deficient mouse revealed an increase in total plasma cholesterol. Further investigation demonstrated, however, that the greatest increase did not occur in LDL cholesterol, but in the VLDL fraction. The atherogenic properties of these particles is largely unknown and currently the subject of much debate and research. We therefore believe that the hypercholesterolemia in this mouse model does not preclude the presence of lipid-independent mechanisms which likely contribute to the development of atherosclerotic disease.

There are a number of other transgenic mice which have been recently developed and show promise in the study of atherosclerosis. These include the apolipoprotein B transgenic mouse [73, 74] as well as the LDL receptor deficient mouse [75]. Both of these animals develop high plasma levels of LDL cholesterol and severe atherosclerotic lesions in response to a high-fat diet. This "human-like" lipid profile makes these mice potentially interesting models for the study of diabetic vascular disease.

AGE-RAGE in cardiovascular disease

Another objective of this study was to investigate the possible role of AGE formation and accumulation in the development of diabetic cardiovascular disease. This animal model of accelerated atherosclerosis in diabetes provides us with a unique tool to investigate the contribution of AGEs in vivo. Under conditions of sustained hyperglycemia, AGE formation was increased, as measured in liver extracts from diabetic mice. We also observed accumulation of AGEs in the diabetic vessel wall by immunohistochemical analysis of aortic tissue sections. At the same time we also observed enhanced expression of the receptor, RAGE, in the endothelium and smooth muscle cells of the aorta after only 6 weeks of diabetes, a timepoint characterized by the presence of accelerated atherosclerotic lesions. These data taken together are consistent with the hypothesis that AGE formation and accumulation in sustained hyperglycemia may contribute to the pathogenesis of accelerated atherosclerosis via RAGE-mediated mechanisms.

These observations and this experimental system lead to many possible avenues of inquiry. What is the identity of the AGE-modified proteins? What are the pathways that

are activated by the binding of AGE to RAGE? And more importantly, is this AGE-RAGE interaction critical to the development of diabetic vascular disease? And can we prevent diabetic complications with therapeutic agents which inhibit this interaction? Further studies to investigate these possibilities are currently underway. One experiment involves administering aminoguanidine, a chemical which prevents the formation of AGEs. As a potential therapeutic agent, however, this compound has limited use clinically. By the time many individuals are diagnosed with diabetes, particularly NIDDM, they have been hyperglycemic for a long period of time and have already accumulated AGEs. Aminoguanidine may prevent further AGE formation, but will have no effect on the activity of the long-lived proteins which have already been modified. Thus, another strategy involves inhibiting the binding of AGEs to their receptor. This may be accomplished using soluble RAGE (sRAGE), which consists of the extracellular portion of the AGE receptor. By administering sRAGE, we may competitively inhibit binding of AGEs to their cellular receptor and prevent activation of potentially pathogenic mechanisms. Studies injecting diabetic apolipoprotein E deficient mice with sRAGE are currently being conducted in hopes of preventing or delaying the development and progression of diabetic vascular complications.

Other experiments include creating transgenic mice with a deletion or cell-specific overexpression of RAGE. These mice may then be crossed with atherosclerosis-prone mice, to observe the effects on the development of disease. Such studies are currently underway in our laboratory.

AGE-RAGE in other diabetic vascular complications

In addition to its role in cardiovascular disease, AGEs have been implicated in a variety of diabetic complications throughout the microvasculature as well. Data from the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) [76] demonstrate a strong correlation between hyperglycemia and the incidence and progression of microvascular disease such as diabetic retinopathy and nephropathy. Immunostaining of renal tissue in patients with diffuse diabetic glomerulosclerosis reveals extensive deposition of AGEs in the vessel wall and Bowman's capsule [77]. Additionally, aminoguanidine has been shown in diabetic rats to prevent the thickening of glomerular basement membrane and trapping of IgG molecules seen in diabetic renal disease [78].

Another important diabetic complication in which AGEs are likely to play a role is in the development of proliferative diabetic retinopathy (PDR), the leading cause of blindness over the age of 65. AGEs have been detected in the vitreous of human diabetic subjects with PDR [Park, L unpublished observations]. They have also been identified the streptozotocin-treated diabetic rat [79]. This research has shown that retinal vessels in these animals demonstrate the characteristic fluorescence of AGEs which correlate with the degree of background retinopathy. Interestingly, treatment with aminoguanidine decreases the level of detectable AGEs and inhibits progression of this disease.

These data indicate that AGEs likely play a critical role in the development of accelerated disease throughout the micro- and macrovasculature of diabetic patients. By investigating their interaction with the cellular receptor, RAGE, and the subsequent pathogenic pathways, we may identify new strategies for possible therapeutic intervention in preventing the complications of diabetic disease.

References

1. Vergely, P., *De l'angine de poitrine dans ses rapports avec le diabète.* Gaz Hebd Med Chir (Paris), 1883. **20**: p. 364-368.

2. Center for Economic Studies in Medicine. *Direct and Indirect Cost of Diabetes in the United States in 1987.* 1988, Alexandria, VA: American Diabetes Association.

3. Marble, A., P. White, and R.F. Bradley, eds. *Joslin's Diabetes Mellitus.* 11th ed. . 1971, Lea & Febiger: Philadelphia. 227-228.

4. Steiner, G., *Atherosclerosis, the major complication of diabetes.* Adv Exp Med Biol, 1985. **189**: p. 277-297.

5. Manson, J.E., *et al.*, *A prospective study of maturity-onset diabetes mellitus and risk of coronary heart disease and stroke in women.* Archives of Internal Medicine, 1991. **151**: p. 1141-1147.

6. Uusitupa, M.I.J., *et al.*, *5-year incidence of atherosclerotic vascular disease in relation to general risk factors, insulin level, and abnormalities in lipoprotein composition in non-insulin-dependent diabetic and non-diabetic subjects.* Circulation, 1990. **82**: p. 27-36.

7. Robertson, W.B. and J.B. Strong, *Atherosclerosis in persons with hypertension and diabetes mellitus.* Laboratory Investigation, 1968. **18**: p. 538-551.

8. Fein, F.S., *Heart disease in diabetes.* Cardiovascular Reviews and Reports, 1982. **3**: p. 877-893.

9. Kannel, W.B. and D.L. McGee, *Diabetes and cardiovascular disease: The Framingham Study.* Journal of the American Medical Association, 1979. **241**: p. 2035-2038.

10. Barrett-Connor, E. and D.L. Wingard, *Sex differential in ischemic heart disease mortality in diabetics: A prospective population-based study.* American Journal of Epidemiology, 1983. **118**: p. 489-496.

11. Hayden, S., *et al.*, *Sex differences in coronary heart disease among diabetics in Evans County, Georgia.* Journal of Chronic Disease, 1980. **33**: p. 265-273.

12. Waller, B.F., *et al.*, *Status of the coronary arteries at necropsy in diabetes mellitus with onset after age 30 years: analysis of 229 diabetic patients with and without clinical evidence of coronary heart disease and comparison to 183 control subjects.* American Journal of Medicine, 1980. **69**: p. 498-506.

13. Hamby, R.I., *et al.*, *Reappraisal of the role of the diabetic state in coronary artery disease.* Chest, 1976. **2**: p. 251-257.

14. Crall, F.V.J. and W.C. Roberts, *The extramural and intramural coronary arteries in juvenile diabetes mellitus: analysis of nine necropsy patients aged 19 to 38 years with onset of diabetes before age 15 years.* American Journal of Medicine, 1978. **64**: p. 221-230.

15. Pyörälä, K., M. Laakso, and M. Uusitupa, *Diabetes and atherosclerosis: an epidemiologic view.* Diabetes Metabolism Review, 1987. **3**: p. 463-524.

16. Wilson, P.W.F., W.B. Kannel, and K.M. Anderson, *Lipids, glucose intolerance and vascular disease: The Framingham Study.* Monogram of Atherosclerosis, 1985. **13**: p. 1-11.

17. Rosenstock, J., *et al.*, *Reduction in cardiovascular risk factors with intensive diabetes treatment in insulin-dependent diabetes mellitus.* Diabetes Care, 1987. **10**: p. 729-734.

18. West, K.M., *et al.*, *The role of circulating glucose and triglyceride concentrations and their interactions with other "risk factors" as determinants of arterial disease in nine diabetic population samples from the WHO multinational study.* Diabetes Care, 1983. **6**: p. 361-369.

19. Rivellese, A., *et al.*, *Presence of very low density lipoprotein compositional abnormalities in type 1 (insulin-dependent) diabetic patients: effects of blood glucose optimisation.* Diabetologia, 1988. **31**: p. 884-888.

20. Howard, B.V., *Lipoprotein metabolism in diabetes mellitus.* Journal of Lipid Research, 1987. **28**: p. 613-628.

21. Fisher, W.R., *Heterogeneity of plasma low density lipoproteins: Manifestations of the physiologic phenomenon in man.* Metabolism, 1983. **32**: p. 283-291.

22. Kissebah, A.H., *Low density lipoprotein metabolism in non-insulin-dependent diabetes mellitus.* Diabetes Metabolism Review, 1987. **3**: p. 619-651.

23. Laakso, M., *et al.*, *Association of low HDL and HDL_2 cholesterol with coronary heart disease in noninsulin-dependent diabetics.* Arteriosclerosis, 1985. **5**: p. 653-658.

24. Christlieb, A., *Diabetes and hypertensive vascular disease: mechanisms and treatment.* American Journal of Cardiology, 1973. **32**: p. 592-606.

25. Wingard, D.L., *et al.*, *Clustering of heart disease risk factors in diabetic compared to nondiabetic adults.* American Journal of Epidemiology, 1983. **117**: p. 19-26.

26. Després, J.-P., *et al.*, *Regional distribution of body fat, plasma lipoproteins, and cardiovascular disease.* Arteriosclerosis, 1990. **10**: p. 497-511.

27. Kannel, W.B. and D.L. McGee, *Diabetes and glucose tolerance as risk factors for cardiovascular disease: The Framingham Study.* Diabetes Care, 1979. **2**: p. 120-126.

28. Manson, J.E., *et al.*, *A prospective study of maturity-onset diabetes mellitus and risk of coronary heart disease and stroke in women.* Archives of Internal Medicine, 1991. **151**: p. 1141-1147.

29. Butler, W.J., *et al.*, *Mortality from coronary heart disease in the Tecumseh Study: Long term effect of diabetes mellitus, glucose tolerance and other risk factors.* American Journal of Epidemiology, 1985. **121**: p. 541-547.

30. Pan, W., -H., *et al.*, *Relationship of clinical diabetes and asymptomatic hyperglycemia to risk of coronary heart disease mortality in men and women.* American Journal of Epidemiology, 1986. **123**: p. 504-516.

31. The Diabetes Control and Complications Trial Research Group, *The effect of intensive diabetes treatment on the development and profession of long-term complications in insulin-dependent diabetes mellitus.* New England Journal of Medicine, 1986. **329**: p. 977-986.

32. The Diabetes Control and Complications Trial Research Group, *Effect of Intensive Diabetes Management on Macrovascular Events and Risk Factors in the Diabetes Control and Complications Trial.* The American Journal of Cardiology, 1995. **75**: p. 894-903.

33. Ross, R., *The pathogenesis of atherosclerosis - an update.* New England Journal of Medicine, 1986. **314**: p. 488-500.

34. Cybulsky, M. and G. M., *Endothelial expression of mononuclear leukocyte adhesion molecule during atherogenesis.* Science, 1991. **251**: p. 788-791.

35. Ruoslahti, E., *Integrins.* Journal of Clinical Investigation, 1991. **87**: p. 1-5.

36. Gerity, R.G., *The role of the monocyte in atherogenesis. Transition of blood-borne monocytes into form cells in fatty lesions.* American Journal of Pathology, 1981. **103**: p. 181.

37. Valente, A.J., *et al.*, *Characterization of monocyte chemotactic protein-I binding to human monocytes.* Biochem Biophys Res Commun, 1991. **176**: p. 309-314.

38. Hansson, G.K., *et al.*, *Immune mechanisms in atherosclerosis.* Arteriosclerosis, 1989. **9**: p. 567-578.

39. Goldstein, J.L., *et al.*, *Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition.* Proceedings of the National Academy of the Sciences USA, 1979. **76**: p. 333-337.

40. Fogelman, A.M. and e. al, *Malondialdehyde alteration of low-density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages.* Proceedings of the National Academy of the Sciences USA, 1980. **77**: p. 2214.

41. Khoo, J.C., *et al.*, *Enhanced macrophage uptake of low density lipoprotein after self-aggregation.* Arteriosclerosis, 1988. **8**: p. 348-358.

42. Ross, R., E.W. Raines, and D.F. Bowen-Pope, *The biology of platelet-derived growth factor.* Cell, 1986. **46**: p. 155.

43. Brownlee, M., *Glycation Products and the Pathogenesis of Diabetic Complications.* Diabetes Care, 1992. **15**(12): p. 1835-1842.

44. Ruderman, N.B., J.R. Williamson, and M. Brownlee, *Glucose and diabetic vascular disease.* FASEB, 1992. **6**: p. 2905-2914.

45. Tanaka, S., *et al.*, *Glycation induces expansion of the molecular packing of collagen.* Journal of Molecular Biology, 1988. **203**: p. 495-505.

46. Monnier, V.M. and A. Cerami, *Nonenzymatic glycosylation and browning of proteins in vivo*, in *The Maillard Reaction in Foods and Nutrition*, G.R. Waller and M.S. Feather, Editors. 1983, American Chemical Society: Washington, D.C. p. 431-439.

47. Dunn, J.A., *et al.*, *Age-dependent accumulation of N-(carboxymethyl)-lysine and N-(carboxymethyl)-hydroxylysine in human skin collagen.* Biochemistry, 1991. **30**: p. 1205-1210.

48. Dyer, D., *et al.*, *Formation of pentosidine during nonenzymatic browning of proteins by glucose.* Journal of Biology and Chemistry, 1991. **266**: p. 11654-11660.

49. Schleicher, E., T. Deufel, and O.H. Wieland, *Non-enzymatic glycosylation of human serum lipoproteins.* FEBS Letters, 1981. **129**: p. 1-4.

50. Witztum, J.L., *et al.*, *Nonenzymatic glycosylation of low-density lipoprotein alters its biologic activity.* Diabetes, 1982. **31**: p. 283-291.

51. Lopes-Virella, M.F., *et al.*, *Glycosylation of low-density lipoprotein enhances cholesteryl ester synthesis in human monocyte-derived macrophages.* Diabetes, 1988. **37**: p. 550-557.

52. Vaughn, M., G. Virella, and M.F. Lopes-Virella, *Diabetes, autoimmunity, and arteriosclerosis.* Clin Immunol Immunopathol, 1989. **52**: p. 414-420.

53. Lyons, T.J. and L. Kennedy, *Non-enzymatic glycosylation of skin collagen in patients with limited joint mobility.* Diabetologia, 1985. **28**: p. 2-5.

54. Kent, M.J.C., N.D. Light, and A.J. Bailey, *Evidence for glucose-mediated covalent cross-linking of collagen after glycosylation in vitro.* Biochemistry Journal, 1985. **225**: p. 745-752.

55. Takeda, H., *et al.*, *Functional changes of cultured endothelial cells induced by glycated extracellular matrix.* Diabetes, 1991. **40 (suppl 1)**: p. A492.

56. Brownlee, M., S. Pongor, and A. Cerami, *Covalent attachment of soluble proteins by nonenzymatically glycosylated collagen: role in the in situ formation of immune complexes.* J Exp Med, 1983. **158**: p. 1739-1744.

57. Vlassara, H., M. Brownlee, and A. Cerami, *High-affinity receptor-mediated uptake and degradation of glucose-modified proteins: a potential mechanism for the removal of senescent macromolecules.* Proceedings of the National Academy of the Sciences USA, 1985. **82**: p. 5588-5592.

58. Vlassara, H., *et al.*, *Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling.* Science, 1988. **240**: p. 1546-1548.

59. Kirstein, M., *et al.*, *Receptor-specific induction of insulin-like growth factor I in human monocytes by advanced glycosylation end product-modified proteins.* Journal of Clinical Investigation, 1992. **90**: p. 439-446.

60. Esposito, C., *et al.*, *Endothelial receptor-mediated binding of glucose modified albumin is associated with increased monolayer permeability and modulation of cell surface coagulant properties.* J Exp Med, 1989. **170**: p. 1387-1407.

61. Neeper, M., *et al.*, *Cloning and Expression of a Cell Surface Receptor for Advanced Glycosylation End Products of Proteins.* The Journal of Biological Chemistry, 1992. **267**(21): p. 14998-15004.

62. Schmidt, A.M., *et al.*, *Receptor for advanced glycation end products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins.* Proceedings of the National Academy of Sciences USA, 1994. **91**: p. 8807-8811.

63. Schmidt, A.M., *et al.*, *Regulation of human mononuclear phagocyte migration by cell surface-binding proteins for advanced glycation end products.* Journal of Clinical Investigation, 1993. **91**: p. 2155-2168.

64. O'Brien, K., *et al.*, *Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques: Implications for the mode of progression of advanced coronary atherosclerosis.* Journal of Clinical Investigation, 1993. **92**: p. 945-981.

65. Li, H., *et al.*, *An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium.* Arteriosclerosis and Thrombosis, 1993. **13**: p. 197-204.

66. Schmidt, A.M., *et al.*, *Advanced Glycation Endproducts Interacting with Their Endothelial Receptor Induce Expression of Vascular Cell Adhesion Molecule-1 (VCAM-1) in Cultured Human Endothelial Cells and in Mice.* Journal of Clinical Investigation, 1995. **96**: p. 1395-1403.

67. Paigen, B., *et al.*, *Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice.* Arteriosclerosis, 1990. **10**: p. 316-323.

68. Zhang, S.H., *et al.*, *Spontaneous Hypercholesterolemia and Arterial Lesions in Mice Lacking Apolipoprotein E.* Science, 1992. **258**: p. 468-?

69. Plump, A.S., *et al.*, *Severe Hypercholesterolemia and Atherosclerosis in Apolipoprotein E-Deficient Mice Created by Homologous Recombination in ES Cells.* Cell, 1992. **71**: p. 343-353.

70. Paigen, B., *et al.*, *Variation in susceptibility to atherosclerosis among inbred strains of mice.* Atherosclerosis, 1985. **57**: p. 65-73.

71. Nishina, P., J. Verstuyft, and B. Paigen, *Synthetic low and high fat diets for the study of atherosclerosis in the mouse.* Journal of Lipid Research, 1990. **31**: p. 859-869.

72. Kunjathoor, V., D. Wilson, and R. LeBoeuf, *Increased Atherosclerosis in Streptozotocin-Induced Diabetic Mice.* Journal of Clinical Investigation, 1996. **97**(7): p. 1767-1773.

73. Callow, M., *et al.*, *Expression of human apolipoprotein B and assembly of lipoprotein(a) in transgenic mice.* Proceedings of the National Academy of the Sciences USA, 1994. **91**: p. 2130-2134.

74. Linton, M., *et al.*, *Transgenic mice expressing high plasma concentrations of human apolipoprotein B100 and lipoprotein (a).* Journal of Clinical Investigation, 1993. **92**: p. 3029-3037.

75. Ishibashi, S., *et al.*, *Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery.* Journal of Clinical Investigation, 1993. **92**: p. 883-893.

76. Klein, R., *Hyperglycemia and Microvascular and Macrovascular Disease in Diabetes.* Diabetes Care, 1995. **18**(2): p. 258-268.

77. Schmidt, A.M., *et al.*, *Cellular Receptors for Advanced Glycation End Products: Implications for Induction of Oxidant Stress and Cellular Dysfunction in the Pathogenesis of Vascular Lesions.* Arteriosclerosis and Thrombosis, 1994. **14**(10): p. 1521-1528.

78. Brownlee, M., A. Cerami, and H. Vlassara, *Advanced glycosylation end-products in tissue and the biochemical basis of diabetic complications.* New England Journal of Medicine, 1988. **318**: p. 1315-1321.

79. Hammes, H., *et al.*, *Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy.* Proceedings of the National Academy of the Sciences USA, 1991. **88**: p. 11555-11558.

HARVEY CUSHING / JOHN HAY WHITNEY
MEDICAL LIBRARY

MANUSCRIPT THESES

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS DATE